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ADVANCED AGENT PROGRAM TOXICOLOGICAL SCREENING METHODS

Stephanie R. Skaggs
Robert E. Tapscott
Everett W Heinonen

Center for Global Environmental Technologies
NEW MEXICO ENGINEERING RESEARCH INSTITUTE
The University of New Mexico
Albuquerque, New Mexico 87131-4339

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Stephanie R. Skaggs, Robert E. Tapscott, and Everett W. Heinonen

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139 Barnes Drive Suite 2
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POC: Dr C.Kibert, WL/FIVCTU
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13. ABSTRACT (Maximum 200 words)

Halon 1211 has been widely used throughout the United States Air Force (USAF) as a streaming agent to extinguish fires. Because it is a depleter of stratospheric ozone, however, its production has been halted. The USAF has been performing research to identify potential replacements. Most "first-generation" candidates, those chemicals that were readily available and had significant toxicological information, are not as effective as Halon 1211, and many have environmental and toxicological drawbacks. Consequently, the USAF has initiated a search for advanced streaming agents. Technology review have identified promising chemical families; however, many of these have insufficient toxicity information to allow a risk evaluation. Detailed toxicity testing is expensive and time-consuming. This document identifies promising methods for inexpensive screening and ranking of chemical families and chemicals within those families that have potential as Halon 1211 substitutes. These tests include acute toxicity or lethality; hepatoxicity; teratogenicity; cardiac sensitization; and genetic toxicity (mutagenicity). These methods cannot replace whole-animal testing as required by regulatory agencies, but can be used as a screening technique to reduce the cost of testing large numbers of chemicals. A second toxicity screening approach uses Quantitative Structure-Activity Relationship (QSAR) analyses.

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LIST OF ABBREVIATIONS

AAWG	Advanced Agent Working Group
ACGIH	American Conference of Governmental Industrial Hygienists
AD_{50}	concentration at which 50 percent of test animals experience anesthesia
AEL	Acceptable Exposure Limit
AIHA	American Industrial Hygiene Association
ALC	Approximate Lethal Concentration
CL	Ceiling Level
CNS	central nervous system
CFC	chlorofluorocarbon
CGET	Center for Global Environmental Technologies
DNA	deoxyribonucleic acid
EC	European Community
EPA	Environmental Protection Agency
FC	(per)fluorocarbon
GWP	Global Warming Potential
HBFC	hydrobromofluorocarbon
HCFC	hydrochlorofluorocarbon
HFC	hydrofluorocarbon
HTOC	Halon Technical Options Committee
IDLH	Immediately Dangerous to Life and Health
LC_{LO}	lowest concentration causing death
LC_{50}	concentration required to cause death in 50 percent of an animal test population
LD_{LO}	lowest dose causing death
LD_{50}	dose required to cause death in 50 percent of an animal test population
LFR	linear free-energy relationship
LOAEL	Lowest Observed Adverse Effect Level
LSER	Linear Solvation Energy Relationship
MCI	Molecular Connectivity Index
MRA	multiple regression analysis
NIOSH	National Institute for Occupational Safety and Health

LIST OF ABBREVIATIONS (CONCLUDED)

NMERI	New Mexico Engineering Research Institute
NOAEL	No Observed Adverse Effect Level
ODP	Ozone Depletion Potential
OSHA	Occupational Safety and Health Administration
PEL	Permissible Exposure Limit
PFC	perfluorocarbon
QSAR	Quantitative Structure-Activity Relationship
REL	Recommended Exposure Limit
SNAP	Significant New Alternatives Policy
STEL	Short-Term Exposure Limit
TLV	Threshold Limit Value
UNEP	United Nations Environment Programme
USAF	United States Air Force
VOC	volatile organic compound
WEEL	Workplace Environmental Exposure Limit Guide
WGL	Workplace Guidance Level

LIST OF SYMBOLS

B	biological activity
d	density
E	biological endpoint
E_S	Taft Steric Substituent Constant
f	fraction in a mixture
F	F statistical or variance ratio
k	rate constant
K	equilibrium constant
K_{OW}	octanol-water partition coefficient
M_R	molar refractivity
M	molecular weight
n	refractive index
P	probability
r	correlation coefficient (also r^2)
E	biological endpoint value
s	standard error of measurement
δ	vertex degree
δ^v	valence vertex degree
ΔF	free energy change for a reaction
$\Delta F^{\ddagger}$	free energy of activation
ρ	reaction constant in Hammett equation
π	log K_{OW}
σ	Hammett Constant
σ^*	Taft Polar Substituent Constant
ψ	resonance effect term
X	molecular connectivity index (chi value)
^nX	molecular connectivity index of order n
$^nX^v$	valence molecular connectivity index of order n
[C]	concentration of a chemical species C

PREFACE

This report was prepared by the Center for Global Environmental Technologies (CGET), New Mexico Engineering Research Institute (NMERI), The University of New Mexico, Albuquerque, New Mexico for the Infrastructure Technology Section of Wright Laboratory (WL/FIVCF), Tyndall Air Force Base, Florida under Contract F08635-93-C-0073, NMERI Number 8-31882. This report provides an assessment of toxicological screening methods.

The project Start Date was 5 December 1994, and the End Date was 30 November 1995. The WL/FIVCF Project Officer is Dr. Charles J. Kibert, and the NMERI Principal Investigators are Stephanie R. Skaggs and Robert E. Tapscott.

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EXECUTIVE SUMMARY

A. OBJECTIVE

The objective of the overall advanced agent program is to develop new, highly effective chemicals to replace Halon 1211 in military streaming applications. The objective of the work discussed in this document is a review and an assessment of screening methods to evaluate toxicological properties of halon replacement candidates. These methods include both *in vitro* toxicity testing and Quantitative Structure-Activity Relationship (QSAR) analyses.

B. BACKGROUND

Halon 1211 is widely used throughout the United States Air Force (USAF) as a streaming agent to extinguish fires. Because it is believed to deplete stratospheric ozone, however, its production has been halted. The USAF has been conducting research to identify potential replacements. Most "first-generation" agents, those chemicals that are readily available and have significant toxicological information, are not as effective as Halon 1211. Moreover, many have environmental and/or toxicological drawbacks. Consequently, the USAF has initiated a search for advanced streaming agents. Technology reviews have identified promising chemical families. Unfortunately, many of these chemicals do not possess adequate toxicity information to be able to assess risk in their use. Detailed toxicity testing is expensive and time-consuming, and a methodology to provide inexpensive and timely toxicity date is required.

C. SCOPE

Work to develop advanced halon replacements was initiated in September 1993 under the Advanced Streaming Agent Testing program. The objective of this program, continued under the Advanced Agent Program, is to develop new advanced chemical replacements for Halon 1211 in streaming applications. The scope of this report is a summary of toxicity screening methods available to enable preliminary and economical toxicity assessments of halon replacement candidates.

D. RESULTS

The literature was reviewed for alternative methods to live animal testing, which is costly and requires large numbers of animals. *In vitro* screening methods, involving exposing cells or tissue preparations to the chemicals of interest and examining parameters specific to the tissues, were identified as promising techniques. Methods of using *in vitro* screening for the required toxicity tests are discussed. In addition, Quantitative Structure Activity Relationships (QSAR) provide a methodology for predicting toxicity based on physical properties, molecular structures, and known toxicities of related compounds. The use of both physical descriptors and molecular connectivity indices of QSARs was examined.

E. CONCLUSIONS

In vitro screening methods have been demonstrated for acute toxicity or lethality and hepatotoxicity tests. *In vitro* techniques work less well on tetratogenicity, cardiac sensitization, and genetic toxicity (mutagenicity). QSARs can be used to make predictions of toxicological properties provided sufficient toxicity information is available on related compounds.

F. RECOMMENDATIONS

A literature search for *in vitro* toxicity data for the chemicals of interest and members of similar families should be undertaken. A detailed search should also be made for toxicity data on chemicals identified as potential replacements for halons and on analogs in the same chemical families. Once these data are available, a determination of whether adequate data exist to perform QSAR analyses should be made and the form of the analyses should be established.

SECTION I
INTRODUCTION

Under previous U. S. Air Force (USAF) sponsorship, the search for "first-generation" halon replacement candidates focused on chemicals that were or soon would be readily available and that had a significant amount of known toxicological information (Reference 1). This strategy was adopted for two reasons: (1) available chemicals could be tested at relatively low cost to determine effectiveness in firefighting scenarios, and (2) toxicological testing of candidates is expensive and time consuming. Chemicals developed primarily as chlorofluorocarbon (CFC) replacements were the major focus as first-generation replacements. Relatively large quantities of these replacements were available, and manufacturers were supporting toxicological testing since the chemicals were often being considered for a number of applications (refrigerants, solvents, foam blowing agents) in addition to halon replacements. This strategy proved successful in that a number of Halon 1211 and Halon 1301 replacement candidates were identified that were readily available for testing and that had a significant amount to toxicity testing already performed (References 2-6).

The first-generation agents were all halocarbons—hydrochlorofluorocarbons (HCFC), perfluorocarbons (FC or PFC), and hydrofluorocarbons (HFC). Hydrobromofluorocarbons (HBFC) were also evaluated very early in the program, but were dropped when it became apparent that they would be regulated due to their still significant Ozone Depletion Potentials (ODP). Although the first-generation program was successful in identifying available candidates that did not require toxicological testing, the first-generation halocarbons were not as effective as Halon 1211 and required approximately two to four times more agent. Moreover, some had toxicological and environmental drawbacks. In particular, environmental concerns have led to the following restrictions:

1. HCFCs will eventually be phased out of production due to their non-zero ODP, and some restrictions are already in place in parts of Europe (and to a limited extent in the USA).

The European Community (EC) regulation 3093/94, which entered into force on 1 June 1995, does not allow the use of HCFCs for fire protection.

2. Under the Significant New Alternatives Policy (SNAP) program, the U.S. Environmental Protection Agency (EPA) has applied narrowed use limits to the use of perfluorocarbons. PFCs are fully fluorinated compounds, unlike HCFCs or HFCs, and have several attractive features. They are nonflammable, have low toxicity, are exempt from federal volatile organic compound (VOC) regulations,* and do not contribute to stratospheric ozone depletion. The environmental characteristics of concern, however, are their high Global Warming Potentials (GWPs), which are as much as 12,500 times that of carbon dioxide (for a 100-year time horizon), and their long atmospheric lifetimes of up to 50,000 years† (Reference 7). Although the actual contributions to global warming depend upon the quantities emitted, the long lifetimes make the warming effects of PFCs virtually irreversible. The EPA is allowing the use of PFCs for only selected applications where no other substitute would meet performance or safety requirements.

3. HFCs have been prominent as replacements for ozone depleting substances. Nevertheless, they are receiving increasing attention from environmental organizations. The National Institute of Public Health and Environmental Protection, The Netherlands, has projected a significant increase in greenhouse gas emissions due to the use of HFCs to replace CFCs and HCFCs (Reference 8). Moreover, Denmark has announced that they plan to phase out all hydrofluorocarbons (HFCs) within the next 10 years due to global warming (Reference 9).

Consequently, the USAF has initiated a search for advanced streaming agents. These advanced agents should be superior in firefighting effectiveness and should have minimal negative environmental effects.

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*VOCs are compounds that contribute to the formation of smog and ground-layer ozone and are controlled in the United States under the 1990 Clean Air Act Amendments (Title III).

†For CF_4. Other perfluorocarbons have considerably shorter, but still long, atmospheric lifetimes.

The call for highly effective fire extinguishing agents requires that the search focus outside the chemical families being investigated as CFC replacements. The properties needed for effective fire and explosion protection agents are distinct from those required for effective refrigerants, solvents, and foam blowing compounds. As a result, a candidate survey was performed for the USAF identifying a number of promising chemical families that appear to have desirable firefighting properties (Reference 10). In addition, work by the Advanced Agent Working Group (AAWG), which includes U.S. Air Force, U. S. Army, and U. S. Navy participation, has identified nonhalocarbon compounds (in particular, phosphorus compounds, metal compounds, and silicon compounds) and short atmospheric-lifetime tropodegradable halocarbons as promising halon substitute candidates (Reference 11). A series of reports, including toxicological information, has been written on several of these families (References 12-16).

Unfortunately, most of the chemicals identified do not possess sufficient toxicity information to be able to evaluate their toxic potentials, and many of the candidates will require toxicity testing. Since toxicological testing is costly, screening methods that can be used to perform a preliminary assessment of the toxicity of a large number of agents would be of great benefit. Of course, more rigorous toxicity tests will likely be required to confirm the toxic effects of selected promising candidates and to satisfy regulatory authorities. Nonetheless, screening methodologies will allow the downselection of the most promising halon replacements.

Two methods can provide preliminary toxicity screening data without the time and expense of live animal (*in vivo*) testing. These techniques are *in vitro* testing and Quantitative Structure-Activity Relationship (QSAR) analyses. While neither can fulfill the requirements of live animal testing required by the EPA and other organizations, the use of either may identify those compounds that can be expected to have low toxicity and should receive priority for additional study. These methods are discussed in Sections III and IV of this report. Prior to this discussion, a survey of toxicity parameters is presented in the following section.

SECTION II

TOXICITY PARAMETERS

The following is an overview of toxicity parameters. Much of this has been abstracted from reports authored by one of the authors of the present report (References 15 and 17), and is based on information compiled by the principal author, Stephanie R. Skaggs.*

A. ACUTE TOXICOLOGICAL INDICES

Table 1 contains a summary of the acute toxicological indices that are discussed in more detail in the following text.

TABLE 1. ACUTE TOXICOLOGICAL INDICES.

	Exposure limit	Definition
ALC	Approximate Lethal Concentration	The approximate concentration considered to cause death, similar to LC_{LO}. Often used in place of LC_{50} when making assessments.
LC_{50}	Lethal Concentration-50%	Concentration causing death in 50% of an animal test population.
LC_{LO}	Lethal Concentration-Low	The lowest observed lethal concentration.
LD_{50}	Lethal Dose-50%	Dose causing death in 50% of an animal test population.
LD_{LO}	Lethal Dose-Low	The lowest observed lethal dose.
AD_{50}	Anesthetic Dose-50%	Dose causing anesthesia in 50% of an animal test population.
LOAEL	Lowest Observed Adverse Effect Level	The lowest exposure level that has been observed to cause an adverse effect. For inhalation of halocarbons, the effect is usually cardiac sensitization.
NOAEL	No Observed Adverse Effect Level	The highest exposure level that has been observed to cause no adverse effect. For inhalation of halocarbons, the effect is usually cardiac sensitization.

*Tapscott, R. E., and Skaggs, S. R., "Alternate Agents," Meeting of the National Association of Fire Equipment Distributors (NAFED), Sectional Conference, Las Vegas, Nevada, 10-11 March 1995, unpublished.

1. Lethality

The LC_{50} is defined as the concentration required to cause death in 50 percent of an animal test population. The ALC value, first established by DuPont but now used by other chemical manufacturers, approximates the lowest concentration that causes death (LC_{LO}). Thus, it is lower than the LC_{50} value. The ALC value is often used in place of the LC_{50} in assessing safety.

2. Anesthesia

Anesthesia is the condition of loss of consciousness, usually coupled with the loss of response to pain and other stimuli. General anesthesia results from a depression of the central nervous system (CNS), which can be exerted by a wide range of chemicals. Anesthetic potency of chemicals is tested in animals by observing decreases in righting reflex (ability to stand up after being knocked over) or diminished response to foot or tail shock. The AD_{50} is the calculated value corresponding to the concentration at which 50 percent of the test animals experience anesthesia. In AD_{50} experiments, anesthesia is defined as loss of the righting reflex or lack of response to shock. Anesthetic potency or mild CNS depression can also be observed in humans using performance decrement studies. Structure-activity relationships have been developed for predicting anesthetic effects (Reference 18).

3. Cardiac Sensitization

Cardiac sensitization is the term used for the sudden onset of cardiac arrhythmias caused by a sensitization of the heart to epinephrine (adrenaline) in the presence of some concentration of a chemical. Many, if not all, halocarbons are cardiotoxins (Reference 19). Halocarbon vapors enter the arterial blood following inhalation and, even with adequate oxygenation, cause ventricular premature beats, ventricular bigeminy, and ventricular tachycardia. Asphyxia increases the problem. In many cases, the compounds also depress myocardial contractility.

When comparing concentrations necessary to elicit acute toxic responses such as anesthesia, cardiac sensitization, or lethality, cardiac sensitization usually occurs at a lower

concentration for halocarbons than other acute toxicity endpoints. Therefore, regulatory and standard-making authorities have used inhalation cardiac sensitization thresholds as the criterion for determining acceptability for use in areas where human occupancy may occur. The phenomenon of cardiac sensitization is particularly important in firefighting because under the stress of the fire event, higher levels of epinephrine are secreted by the body, increasing the possibility of sensitization.

The threshold level is the lowest concentration at which cardiac sensitization occurs. No definitive rule exists indicating the number of animals that must experience a marked response to determine the threshold value. In most cases, even one animal experiencing a marked response constitutes establishment of a threshold value. This level is also called the Lowest Observed Adverse Effect Level (LOAEL). The highest concentration at which no marked responses occur is called the No Observed Adverse Effect Level (NOAEL). Although it is not known with certainty whether the LOAEL and NOAEL in dogs accurately represent these values in humans, the dog is the preferred animal model for determining cardiac physiology.

LOAEL and NOAEL concentrations entail measurement of cardiotoxic effects in animals made sensitive to these effects by the administration of epinephrine. The administered epinephrine doses are just below the concentration at which epinephrine alone would cause cardiotoxicity in the experimental animal and are approximately ten times greater than the concentration a human would be likely to secrete under stress. Thus, LOAEL and NOAEL values are conservative even in high-stress situations.

B. SUBCHRONIC AND CHRONIC TESTS

1. 90-Day Subchronic Toxicity Test

The 90-day subchronic toxicity test is an assay that determines pathological changes due to repeated and prolonged chemical exposure. Subchronic toxicity testing provides the basis for developing industrial exposure standards.

2. Chronic Toxicity Testing

Chronic toxicity tests are conducted over the greater part of the animal's lifespan (1.5 to 2 years in mice and 2 or more years in rats), starting at weaning. Daily exposure to the test agent occurs. The principal endpoint is tumor formation, as determined by histological exam.

3. Carcinogenicity Screening

Chemical carcinogenesis is usually the result of long-term exposure to a chemical that may occur generally during industrial processing and handling. To determine the potential carcinogenicity of an agent, genotoxicity (mutagenicity) screening tests are often performed. Positive mutagenicity results alert toxicologists to the possibility of carcinogenesis and indicate the need for subchronic exposure testing to develop industrial exposure standards. Although many tests are used, the following genotoxicity tests are the most common.

a) Ames Test

The Ames test, an *in vitro* test for mutagenicity and, by implication, carcinogenicity, uses mutant strains of bacterium *Salmonella typhimurium* as a preliminary screen for carcinogenic potential (Reference 20). A number of assays comprise the Ames test, and positives indicate that a mutation in the genetic material has occurred. Mutagenic and presumed carcinogenic materials cause genetic mutations that allow the bacterial strains to grow in a histidine-free medium.

b) Mouse Lymphoma Test

The mouse lymphoma test, also an *in vitro* screening test, uses cell cultures of mouse lymphoma cells. The mutagenic potential of a material is tested by observing the ability to confer resistance within this cell line to normally toxic agents. Mutations in the genetic material allow the cells to grow in the presence of other known toxic materials (purines, pyrimidines, or ouabain). Promutagens (mutagenic agents that require metabolic activation) can also be identified.

c) Mouse Micronucleus Test

The mouse micronucleus test, an *in vivo* test, determines the potential of a chemical to cause chromosome breakage or interference with normal cell division. The test entails exposing live mice to the test material, then removing premature red blood cells from the bone marrow, and observing the cells for the presence of chromosome fragments or the lack of signs of normal cell division. This test is not considered the most sensitive test for chromosomal aberrations.

C. EXPOSURE LIMITS

Four major noncommercial organizations establish or recommend occupational exposure limits. The National Institute for Occupational Safety and Health (NIOSH) and the Occupational Safety and Health Administration (OSHA) are governmental organizations. Standards established under OSHA are enforceable; however, NIOSH only sets recommended occupational exposure limits. Non-governmental organizations establishing exposure limits are the American Conference of Governmental Industrial Hygienists (ACGIH) and the American Industrial Hygiene Association (AIHA). Table 2 gives the various exposure limits that have been established. Note that most of these levels are neither used extensively nor well developed. The only ones with significant use by industrial hygienists are the PEL, the WEEL, and the TLV, which are the appropriate upper exposure limit for safe handling over a lifetime of occupational exposure (e.g., industrial processing, rather than firefighting). The Acceptable Exposure Limit (AEL), which is widely cited, was originally used by DuPont; however, it is now given by a number of other commercial organizations.

TABLE 2. EXPOSURE LIMIT DEFINITIONS.

	Exposure limit	Establishing organization	Definition
	Long-Term Exposures		
AEL	Acceptable Exposure Limit	Commercial	
PEL	Permissible Exposure Limit	OSHA	Enforceable 8-hr Time-Weighted Average (TWA) exposure limit for airborne substances intended to reduce a significant risk of health or functional capacity impairment.
REL	Recommended Exposure Limit	NIOSH	Similar to TLV values.
TLV	Threshold Limit Value	ACGIH	TWA exposure limits similar to PEL values.
WEEL	Workplace Environmental Exposure Limit Guide	AIHA	Similar to TLV values.
WGL	Workplace Guidance Level	EPA	8-hr day TWA value analogous to PEL values.
	Short-Term Exposures		
CL	Ceiling Level	OSHA	Enforceable exposure level that cannot be exceeded for any time period.
STEL	Short-Term Exposure Limit	OSHA	Enforceable 15-min TWA exposure that should not be exceeded at any time during a work day.
IDLH	Immediately Dangerous to Life and Health	NIOSH	Maximum concentrations from which one could escape within 30 min without experiencing escape-impairing or irreversible health effects.
EGL	Emergency Guidance Level	EPA	Applies to short-term exposure of 15 or 30 min and is similar to the IDLH.

SECTION III
TOXICOLOGICAL SCREENING METHODS

The literature was searched for alternative methods to live animal testing. The basic methods for *in vitro* tests involve exposing cells or tissue preparations to chemicals of interest and then examining parameters specific to the tissues to determine the effect of the exposure. These methods are most successful when the agents are either liquids or can be dissolved in relatively benign solvents (aqueous solutions are best) such that the tissue preparations or cells can be exposed to known quantities. Problems arise when the agents of interest are gases, especially if they are not water soluble. Exposure concentrations are much more difficult to determine in these situations, and uncertainties exist about the relationship to doses in whole-animal testing.

In vitro screening methods hold great promise for diminishing the cost of determining the relative toxicities of a large number of chemicals. However, these methods should not be presumed to replace whole-animal testing to determine absolute toxicities, especially as required by regulatory agencies. Rather, these screening technologies are useful in testing and ranking a wide variety of chemical substances on the basis of toxic potential. Even though screening technologies reduce the cost of testing a large number of chemicals, fairly large expenditures may be necessary to determine the mechanism by which chemicals are toxic so that appropriate screening tests are designed. Validation of the screening tests to whole-animal systems can also be costly in some cases.

A. ACUTE TOXICITY OR LETHALITY

Alternatives to whole vertebrate animal toxicity testing for acute lethality exist. The methods developed include *in vitro* techniques, avian embryo assays, isolated enzyme function studies, invertebrate animal testing, and mathematical modeling (e.g., QSARs). A major problem with *in vitro* techniques is that unless the mechanism of lethality is well understood (which is rare for never-tested chemicals), these techniques rarely mimic results of whole-animal tests (Reference 21). Knowledge of target organs is a requirement for design of *in vitro* assays,

since selection of isolated cell from an organ resistant to the effects of a chemical will yield erroneous results when compared to whole-animal tests.

Chicken embryo tests are sometimes used as a model for acute toxicity testing. Published data from the Czechoslovakian Academy of Sciences suggest that their chicken embryotoxicity screening test has a high degree of predictive value when compared to data derived from traditional rodent acute toxicity tests for some classes of chemicals (Reference 22).

B. HEPATOTOXICITY

Hepatotoxicity can be investigated using cultured or isolated liver cells, liver slices, or isolated, perfused whole livers. Human hepatocytes from biopsy perfusion methods have been used in some tests, but animal cells are usually used. For animals cells, tissue from two or three animals is often sufficient to conduct studies that would ordinarily require 20 to 40 animals (Reference 23). The use of isolated hepatocytes offers three advantages: (1) preparations are easily made without sophisticated equipment; (2) a large number of experiments can be performed with the liver of only one animals; and (3) isolated hepatocytes can serve as their own control. A number of publications have described the preparation, properties, and applications of isolated hepatocytes (References 24-26).

In vitro hepatotoxicity studies are performed to identify chemicals that specifically produce toxicity in the liver and to determine the metabolic kinetics of chemicals and the metabolic forms potentially excreted (Reference 23). *In vitro* systems offer the potential of studying liver injury without extrahepatic factors such as absorption, distribution, and extrahepatic metabolism (Reference 27).

C. TERATOGENICITY

Evaluating teratogenicity using *in vitro* systems involves establishing the relationship between *in vivo* indices of toxicological response and the complex process of differential toxicity in the developing organism, especially one as complex as the human fetus (Reference 23). Proposed alternative test systems have ranged from whole organisms—hydra or fruit fly to frog

or rodent embryos—to cell cultures. The use of whole organisms test methods circumvents the problems associated with extrapolation from cell cultures to whole animals. However, these whole-organism systems still present questions about predicting human teratogenicity.

D. CARDIAC SENSITIZATION

The mechanism of cardiac sensitization is not completely understood, making design of a screening method difficult. It is thought that cardiac sensitization is a direct result of a physical-chemical interaction of the sensitizing agent with heart cell membrane structures (Reference 28). This mechanistic hypothesis is derived from the observation that a critical blood concentration of agent is needed to elicit a cardiac sensitization response. Below that critical concentration, the effect does not occur, and the effects are immediately reversible, if they are not been sufficiently severe to cause death, when the sensitizing agent is removed. However, other systemic effects cannot be completely ruled out.

The development of an *in vitro* cardiac sensitization screening method requires the development and validation of a system that retains the essential components of the process of cardiac sensitization. Requirements for an *in vitro* system include (Reference 28) a cardiomyocyte system that

(a) exhibits synchronized and spontaneous contraction,

(b) is responsive to exogenous epinephrine,

(c) exhibits sensitivity to known cardiac sensitizers, and

(d) allows exposure of sensitizing agents to system in a controlled atmosphere.

Under the current state of technology in the field, the establishment of such a system is feasible.

As early as the 1960s, cardiac myocytes were used to study the mechanistic aspects of cardiac metabolism and function (Reference 28). A number of *in vitro* systems using isolated or cultured cardiomyocyte cells, tissue preparations, or excised whole hearts have been established

to investigate cardiac toxicity. In addition, some studies have used subcellular organelles. Much of the research has involved chick embryo myoblast cell cultures. However, questions, which would require an extensive effort to resolve, exist about the extrapolation of results from avian to mammalian cardiac toxicity.

1. Cardiac Cell Preparations

Several preparations have been performed where isolated ventricular myocytes have been isolated or cultured while retaining their spontaneous electrical activity (Reference 28). Cultured neonatal rat heart cell cultures have been used to investigate the effects of quinidine ($C_{20}H_{24}N_2O_2$), an alkaloid drug used to control tachycardia, on cardiac cell function (Reference 29). Sodium flux measurements and myocyte contraction rates demonstrated that quinidine reduced the spontaneous beating rate of the cells in culture, and this effect was related to reduced sodium influx. In some preparations, contractility or "beating" can also maintained. Parameters used to assess cardiomyocyte function include cell lethality, electrical properties, and cell contraction rate.

In vitro cellular model systems have been extensively used to research basic cardiac research activities. Types of *in vitro* cardiac systems are shown in Table 3. Most of these studies, however, were not performed to investigate the toxicological effects of the chemicals. Some were carried out to assess effects of certain drugs on cardiac function, and others were performed to determine specifics of cardiac function itself.

TABLE 3. *IN VITRO* CARDIAC CELL SYSTEMS.

Model	Life stage	Species	Reference
Myocardial cells	Fetal	Human	Goldman (30)
Cardiomyocytes	Neonatal	Rat	Mark and Strasser (31)
Ventricular Cardiomyocytes	Neonatal	Rat	Schanne (32)
Ventricular Cardiomyocytes	Neonatal	Dog	Liu (33)
Cardiomyocytes	Adult	Rat	Vahouny (34)
Cardiomyocytes	Adult	Rabbit	Dani (35)
Cardiomyocytes	Adult	Dog	Youker (36)
Cardiomyocytes	Adult	Feline	Woosley (37)
Ventricular Cardiomyocytes	Adult	Guinea Pig	Failli (38)
Ventricular Cardiomyocytes	Adult	Rat	Eid (39)

Despite the variety of *in vitro* systems available to assess the cardiac toxicity of certain agents, any model selected would have to be characterized with respect to the expression and response of adrenergic receptors (the binding site of epinephrine) and the ion fluxes (sodium and calcium) before cardiac sensitization validation studies could be initiated (Reference 28). Since a relatively large amount of data on cardiac sensitization in dogs exists, dog cardiac myocyte cell preparations would be convenient for validation of *in vitro* results with *in vivo* assessments. Vahouny et al., (Reference 40) described a procedure for isolating dog ventricular myocytes based on their procedure for preparing adult rat heart cells (Reference 34). Dogs cells isolated by this procedure, however, lost their spontaneous beating activity rapidly (within 1 hour), but could be stimulated to contract with high calcium concentrations, ouabain, and epinephrine. Consequently, proper procedures need to established to assure that contractility is maintained in cell preparations.

2. Cardiac Subcellular Organelles

Subcellular organelles, such as mitochondria or sarcolemma, have also been used to investigate mechanisms of cardiac function (Reference 28). Until the mechanism of cardiac

sensitization (i.e., premature ventricular ectopic beats or ventricular fibrillation in response to agents and epinephrine) is elucidated, however, subcellular organelle function is probably not relevant. Basic research on the phenomenon of cardiac sensitization will establish if, for example, the sensitizing agents alter membrane calcium pump activity and/or regulation. Until that time, studies on subcellular organelles should not be emphasized.

3. Isolated Heart Tissue Preparations

Most isolated heart tissue preparations utilize atrial tissue from adult animals. Heart tissue preparations have advantages over isolated cell cultures for electrophysiological studies of *in-situ* signal conduction (Reference 28). Spontaneously beating preparations of rat and guinea pig atrial tissues have been described by a number of researchers (References 41-44).

4. Isolated Heart Preparations

The isolated heart preparations have been used for many years to investigate the regulation of cardiac function (Reference 28). These preparations have the advantage of maintaining the integrated function of the whole heart. Electrophysiological studies can be performed to answer questions relevant to cardiac sensitization by measuring action potential conduction velocities.

E. GENETIC TOXICITY (MUTAGENICITY)

The goal of genetic toxicity testing is to identify chemicals capable of affecting the hereditary components of a living organism at subtoxic concentrations. Mutagenicity is a subclass of genetic toxicity in which the sequence of genes, chromosome structure, or chromosome numbers is changed. Chemicals can be tested for mutagenetic effects by adding known amounts of the chemicals to specific organisms and observing the effect over time. A common *in vitro* test is the Ames test, in which the test chemical is added to mutant strains of the *Salmonella Typhimurium* bacterium, with a mammalian S9 activation component if required. Since the bacterium cannot grow in the test medium without mutation, the amount of bacterium

growth indicates the percentage of the bacterium that have mutated to a form that can grow and thus provides an indication of the mutagenic ability of the test chemical.

Other *in vitro* mutagenicity testing protocols include the mouse lymphoma assay, the Chinese hamster *in vitro* assay, the sister chromatic exchange in CHO cells, and the unscheduled deoxynucleic acid (DNA) synthesis in rat liver primary cell cultures. These protocols are fully described in Reference 45.

F. CARCINOGENICITY

While many short-term tests for determining genotoxicity have been developed, primarily for screening for genetic toxicity, they have not replaced the classic carcinogenicity studies. The relationship between carcinogenicity and genotoxicity is not direct, and genotoxicity studies do not accurately indicate carcinogenicity. Therefore, no screening techniques for carcinogenicity are available above those used in genetic toxicity testing (Reference 45).

SECTION IV

QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIPS

A. BACKGROUND

Quantitative structure-activity relationships have been used for over 100 years to make quick and inexpensive predictions about the toxicity of chemicals (Reference 46). A QSAR is a mathematical model that relates a biological activity (such as toxicity) to chemical, physical, or molecular structure properties of the chemical (Reference 47). Three components for any QSAR are (1) a biological activity that can be quantitatively measured, (2) chemical or physical descriptors or structural features, and (3) a technique used to establish a relationship between the two (Reference 48). For a QSAR to be meaningful, three requirements must be fulfilled. First, the model must be accurate enough to be useful (generally within an order of magnitude). Second, the QSAR must be applied only within the group of chemicals used to define the equation. Finally, the data required to establish the equation must be easily obtained by experiment or available through existing databases. The latter requirement is usually the most difficult to meet. QSAR techniques were originally used for pharmacology, but have more recently been applied to environmental risk assessment and regulation development.

The basis of a toxicological QSAR lies in the statistical establishment of a mathematical relationship between chemical toxicity, as indicated by the biological response of test animals, and certain chemical or physical properties of a group of congeneric (similar) compounds. The mathematical model can then be used to predict the toxicity of untested chemicals in the same group or similar groups based on the properties of the tested chemicals. While the answers provided may not be based on a fundamental understanding of the toxicity process, they often provide valuable screening information on whether chemicals or classes of chemicals would be expected to have acceptable toxicity for specific uses.

The type of toxicity must be considered in using QSAR techniques. For example, reactive (or specific) toxicity is associated with a specific reactive mechanism, such as a chemical reaction with an enzyme or inhibition of a chemical pathway, and is dependent upon

specific structure. Nonreactive (or nonspecific) toxicity is not related to a specific mechanism but rather to the amount of chemical acting upon the cell (Reference 47). Nonreactive toxicity of a chemical is believed to be related to its solubility in lipids, and water solubility or octanol/water partition coefficients (K_{OW}) are often used as properties in QSARs. When used to determine reactive toxicity, these relationships should not be used to determine nonreactive toxicity.

The key to using any of these techniques is to determine a family of chemicals for which biological indices (for example, LD_{50}), as well as physical/chemical properties, molecular structure, or both, are known. By the use of statistical techniques such as multiple regression analysis (MRA), a relationship between the biological indices and property/molecular structure is determined. Chapter 5 of Reference 49 provides an excellent description of the techniques. As an example of the form of a relationship, Equation 1 is used to predict the activity of antitumor triazenes, which attack cancer cells (Reference 50).

$$\log 1/[C] = 0.10\pi - 0.042\pi^2 - 0.31\sigma + 0.18M_R(2,6) + 0.39E_S(R) + 4.12 \qquad (1)$$

In this expression, [C] is the molar concentration of triazene producing an increase in life span in mice of 40 percent; $\pi = \log K_{OW}$, where K_{OW} is the octanol/water partition coefficient; $M_R(2,6)$ is the molar refractivity of substituents in the 2 and 6 position; $E_S(R)$ is the Taft Steric Substituent Constant for the largest R group substituent; and σ is the Hammett Constant. The last two constants are discussed later in this report. This equation is based on 61 compounds. With each set of equations, a correlation coefficient (r or r^2), a standard error of measurement(s), and the F statistic, or variance ratio is determined, indicating the "goodness" of fit of the equation. The F statistic assesses the probability that the correlation is different from randomness, i. e., that the equation is not just based on random numbers. The higher the value of F, the greater the probability that the equation accurately reflects the data. In this example, $r = 0.836$ and $s = 0.191$, indicating a rather poor fit of the data by the equation, but one nonetheless useful in certain circumstances (Reference 51); F is not provided. Most of the work done in QSAR analysis is to develop equations with high correlation coefficients using known chemicals, which can then be used to determine the biological indices of unknowns.

Many parameters can be used in relationships between biological activity and chemicals. These can be classified as property descriptors and topological descriptors (steric and structural). Table 4 lists some of these descriptors (Reference 48).

TABLE 4. PHYSICAL AND STRUCTURAL QSAR DESCRIPTORS.

Type	Example
Property	
General	melting point
	boiling point
	vapor pressure
	dissociation constant
	activation energy
	heat of reaction
	reduction potential
Hydrophobicity	octanol/water partition coefficient, K_{OW}
	R_M coefficient from reverse-phase chromatography
	solubility in water
	parachor
Electronic	Hammett Constant, σ
	Taft Polar Substituent Constant, σ^*
	ionization potential
	dielectric constant
	dipole moment
	hydrogen bonding
Quantum chemical	Molecular Orbit indices
	electron density
	π-bond reactivity
	electron polarizability

TABLE 4. PHYSICAL AND STRUCTURAL QSAR DESCRIPTORS (CONCLUDED).

Type	Example
Steric Descriptors	
Molecular volume	
Molecular weight	
Molecular surface area	
Molar refractivity	
Substructure shape	
Taft Steric Substituent Constant	E_s
Verloop STERIMOL constants	L, B1-B3
Structural Descriptors	
Atom and bond fragments	
Substructures	
Substructure environment	
Number of atoms in group	
Number of rings (in polycyclics)	
Molecular connectivity	

B. PROPERTY RELATIONSHIPS

Under this type of QSAR, measured physical, chemical, or optical properties are used to provide the data for the analysis. Almost any physical chemical property of the chemicals may be used to establish a relationship, although some may be better choices than others. Since many of the physical properties are related, the choice of properties may be limited. Major categories of physical properties include general properties, hydrophobicity, electronic descriptors, and steric effects.

1. General Properties

These properties, such as boiling or melting points, are commonly known for many compounds, and can easily be used in a QSAR analysis.

2. Hydrophobicity

Hydrophobicity, a measure of the lack of water solubility, is commonly used in QSAR work. Neutral, unreactive chemicals exhibit a strong relationship between the hydrophobicity of a compound and the LC_{50}. This is described by the octanol/water partition coefficient, which relates to the ability of a chemical to cross a biological membrane and interact with the cell. The octanol/water partition coefficient is used because the cellular biophase is similar to octanol (n-octyl alcohol, $C_8H_{17}OH$) in physical properties. Hydrophobicity correlates with toxicity not because chemicals that are hydrophobic are more toxic, but because the diffusion through cell walls is greater. When described as log K_{OW} ($= \pi$), where K_{OW} is the distribution of a chemical in the water-octanol system, a relationship emerges between log K_{OW} and log B (some biological activity) that is roughly parabolic. At low values of log K_{OW}, the relationship is direct, but at higher log K_{OW}, the low aqueous solubility will decrease the toxicity of a compound (Reference 52). Since log K_{OW} can be easily and accurately determined for most compounds, it is commonly used in the QSAR process.

3. Electronic Properties

Electronic properties play a part in the specific interaction between a compound and a biological receptor. It is proposed that the difference in the reaction free energies (ΔF) of a compound and a standard (usually differing by the presence or absence of a substituent X), $\Delta\Delta F = \Delta F_X - \Delta F_O$, is given by the sum of contributions from electronic, resonance, and steric effects. This is designated as a linear free-energy relationship (LFR). The primary electronic effect is the polarity, the electron-attracting power of a substituent. Resonance can also be considered as an electronic effect. Using analyses of rate and equilibrium constants based on this concept, one can determine polar, steric, and resonance effects. The effect of a substituent on the electronic properties (e.g., the polar effect) has been characterized by two constants, the Hammett Constant

(σ) and the Taft Polar Substituent Constant (σ^*), both of which are commonly used to develop property-activity relationships.

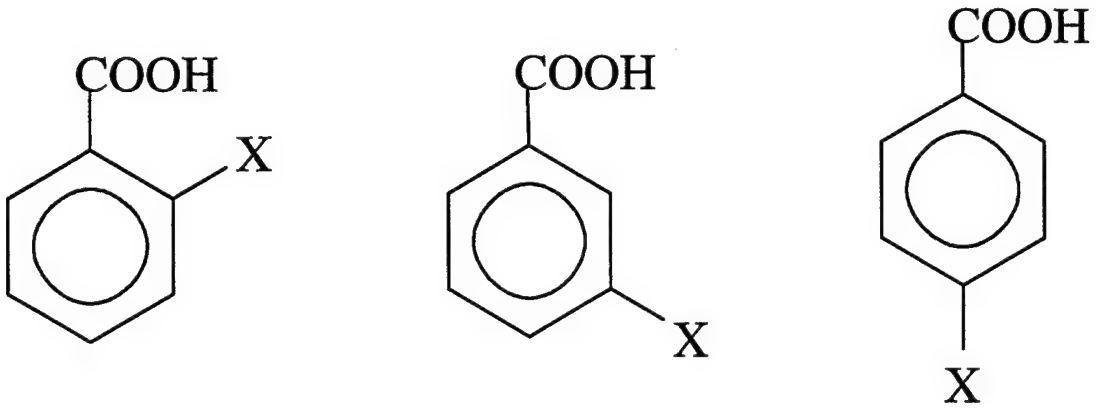
The Hammett Constant relates structure to both equilibrium constants and rate constants for *meta*- and *para*-substituted benzene derivatives (Reference 53). In this case, groups are well separated from each other by a relatively rigid benzene ring and are not expected to interact sterically. Thus, steric effects must be minimal and any variations in rate constant are due primarily to polar (and, for some substituents, resonance) effects. Equation 2 defines the Hammett Constant for a substituent X in the *meta* or *para* position of benzoic acid (C_6H_5COOH). Here K_X and K_O are the ionization constants for substituted and unsubstituted benzoic acid at 25 °C in water. Under other conditions (different solvent, temperature, etc.), a reaction constant ρ must be included (Equation 3).

$$\sigma = \log (K_X/K_O) \tag{2}$$

$$\sigma\rho = \log (K_X/K_O) \tag{3}$$

In *meta* and *para* isomers (in contrast to *ortho* isomers), the substituent is well separated from the carboxylic acid group, minimizing steric interactions (Figure 1). Values of σ have been calculated for many of the common substituents for both the *meta* and *para* positions on the benzene ring. A positive σ value ($K_X > K_O$) for a substituent indicates that the substituent is a stronger electron attractor than hydrogen, and a negative σ value ($K_X < K_O$) indicates that the substituent is a weaker electron attractor. Stronger electron attractors stabilize the product benzoate anion ($C_6H_5COO^-$), thus increasing the acid ionization constant K.

One can apply the Hammett Constants determined from substituted benzoic acid ionizations to equilibrium constants for other reactions, where steric effects are not important, by determination of the appropriate value of ρ and redefining K_x and K_o in Equation 3. One can also apply the Hammett Constants to reaction rates as shown in Equation 4, where k_X and k_O are the rate constants for reactions of a substituted and unsubstituted reactant. (Here, analogous to that done for equilibrium constants, it is assumed that the difference in the reaction free energies



Ortho Meta Para

Figure 1. Isomers of Disubstituted Benzene.

of activation for a compound and a standard, $\Delta\Delta F^{\ddagger} = \Delta F_X^{\ddagger} - \Delta F_O^{\ddagger}$, is given by the sum of electronic, resonance, and steric contributions.) Thus, a plot of log k_X as a function of σ should be linear with a slope of ρ and an intercept log k_O (Equation 5). This allows an estimation of a reaction rate for a given compound given sufficient data for other derivatives to determine ρ and k_O. Again this methodology can only be applied to reactions where steric effects play an insignificant role. Equilibrium constants can be estimated in a similar way.

$$\sigma\rho = \log (k_X/k_O) \tag{4}$$

$$\sigma\rho = \log k_X - \log k_O \tag{5}$$

The reaction constant ρ can be either negative or positive depending on how substituent electron withdrawal or electron induction affects equilibrium or rate constants for a given reaction. A positive value for ρ indicates that electron withdrawing substitutents increase the reaction rate or equilibrium. A negative value indicates facilitation by electron inducing substituents.

The Hammett equation can fail (i.e., σ values can vary for a given substituent) when resonance is possible between a substituent and the functional group involved in the

reaction whose equilibrium or rate constant is under consideration. For example, the σ value determined for a *p*-NO_2 substituent from *p*-nitrobenzoic acid ionization is not the same as that determined for ionization of the *p*-nitroanilinium ion ($O_2NC_6H_4NH_3^+$). In this case resonance across the aromatic system is possible (Figure 2). One can take resonance into account in para-substituted benzene derivatives by introducing a resonance effect term ψ into Equation 4 to give Equation 6.

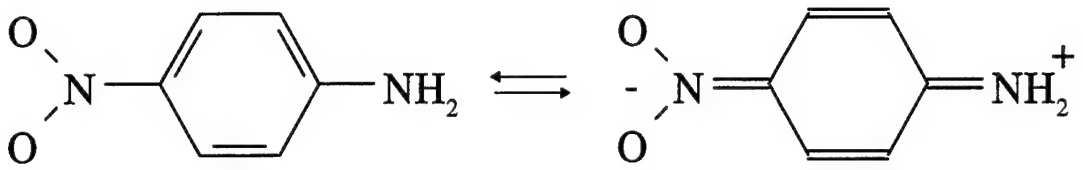


Figure 2. Resonance Structures for *p*-Nitroaniline.

$$\sigma\rho + \psi = \log (k_x/k_o) \tag{6}$$

Like the Hammett Constant (σ), the Taft Polar Substituent Constant (σ*) is a measure of substituent polarity. In this case, substituted aliphatic esters, RCOOR' (rather than benzoic acids) are used, and the values are determined from rates of acidic and basic hydrolyses (Reference 54). In Equation 7, k_x and k_o are the hydrolysis rate constants with the substituent X (R = X) and for a reference standard (R = CH_3), The subscripts "A" and "B" denote acidic and basic conditions, respectively. Unlike the case for *ortho* and *para* isomers of benzoic acid, steric factors are important in aliphatic ester hydrolysis. However, Equation 7 allows one to eliminate the steric (and resonance) contribution, which is assumed to be the same in both acidic and basic hydrolysis of the same compound. The factor of 1/2.48 is introduced in an attempt to put the Taft and Hammett constants on the same scale.

$$\sigma^* = \left(\frac{1}{2.48}\right)[\log (k_X/k_O)_B - \log (k_X/k_O)_A] \tag{7}$$

4. Steric Properties

Steric properties relate to the structure of the molecule and affect the binding of the molecule to a biological site (Reference 48). The Taft Steric Substituent Constant (E_S) (References 55 and 54) is one measure of the steric effect of a substituent in a molecule. This constant is determined from the rate constants (k) for hydrolysis of RCOOR' in acid solution (Equation 8). Here k_X and k_O and the subscript "A" are defined as for Equation 7. E_S values decrease with increasing steric interactions.

In general, steric effects control acid hydrolysis of esters. Base hydrolysis is controlled by both steric and electronic effects. E_S and σ^* are related as shown in Equation 9.

$$E_S = \log (k_X/k_O)_A \quad (8)$$

$$E_S + 2.48\sigma^* = \log (k_X/k_O)_B \quad (9)$$

5. Relationship of Biological Properties to Property Descriptors

In 1964, Hansch proposed Equation 10 (commonly called the Hansch Equation), where B is a biological activity of a member of a series of compounds RX with varying substituents X, and π (= log K_{OW}), σ, and E_S represent the effects of substituents on the hydrophobic, electronic, and steric properties within the series (Reference 56). (Note the resemblance to Equation 1.) The coefficients a, b, c, d, and e are determined by regression analysis. One of more of the descriptors π, σ, and E_S may not be critical in the analysis and may be omitted.

$$\log B = a\pi^2 + b\pi + c\sigma + dE_S + e \quad (10)$$

Numerous programs have been designed for desktop computers to aid in performing required regression analyses. BIOSOFT QSAR-PC: PAR, distributed by BIOSOFT, Cambridge, United Kingdom, is an example of an early program.

C. STRUCTURAL RELATIONSHIPS

The physical descriptors described above are a consequence of the structure of the molecule involved. If structural features that govern the physical properties can be identified, those features themselves can serve as the terms in the relationship to biological functions. Two aspects of the structure can be identified: the topology, which is the identity of the atoms and their connections, and the topography, which is the three-dimensional part of the molecule such as size, shape, volume, and surface area.

1. Molecular Connectivity Indices

Molecular connectivity descriptors allow one to derive a direct relationship between the number of atoms in a compound, together with their formally-bonded and spatial relationships, and a biological activity. This is most commonly accomplished by the use of connectivity indices, which are composites of all structural features. Molecular Connectivity Indices (MCI) associate numerical values with the structure of the molecule. Many properties are directly related to the number and connections of atoms in a series of molecules, and molecular connectivity is a method for developing correlations based on a formal development of molecular connectivity using elementary aspects of graph theory. Reference 57 provides an excellent description of these indices. Structural information encoded in these data can be closely related to physical properties. The following is a brief description of molecular connectivity indices and how they are calculated and used.

Calculation of these indices begins with a creation of a molecular representation in which all bonds not involving hydrogen are represented as lines. All intersections ("vertices") represent atoms. Lines joining the vertices and representing formal bonds are called "edges." Each non-hydrogen atom is designated by a cardinal number, designated as δ, which provides a count of the number of adjacent non-hydrogen atoms. The vertex valence number, δ^v, of atoms in the first two rows of the periodic table is determined by subtracting the number of attached hydrogen atoms (h) from the number of valence electrons (Z^v) (Equation 11). For atoms beyond

the second row of the periodic table, the δ^v values are calculated by Equation 12, where Z is the atomic number. The δ^v values for some common heteroatoms are shown in Table 5.

$$\delta^v = Z^v - h \tag{11}$$

$$\delta^v = \frac{Z^v - h}{Z - Z^v} \tag{12}$$

TABLE 5. EXAMPLES OF VALENCE DELTA VALUES FOR HETEROATOMS.

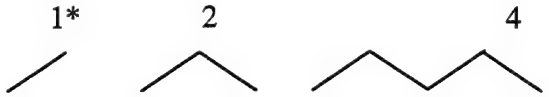
Atom	δ^v
$-NH_2$	3
-NH-	4
$-\overset{\vert}{N}-$ and =N-	5
-OH	5
=O and -O-	6
Cl	0.70
Br	0.25

Each bond is identified by two adjacent non-hydrogen atoms, designated i and j, which form the bond. Subgraphs are also constructed showing the δ and δ^v values for the edges of the graph (1st order subgraphs), adjacent two-edge segments of the graph (2nd order subgraphs), adjacent three-edge segments (3rd order subgraphs, which can contain paths [three successive edges], clusters [three edges joined at a point], or chains [rings]), and higher segments. Table 6 illustrates some of these subgraphs with the numbers giving the orders of the substructures.

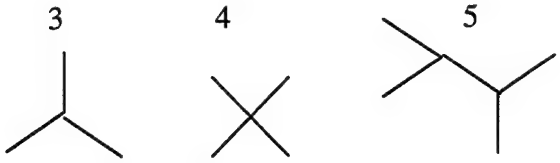
The MCI, represented by the Greek letter chi (X), is computed for each type and order of subgraph into which the molecular graph can be decomposed. The level of molecular connectivity consideration dictates whether δ or δ^v terms are used. MCI connectivity index

TABLE 6. MOLECULAR CONNECTIVITY SKELETON SUBGRAPHS.

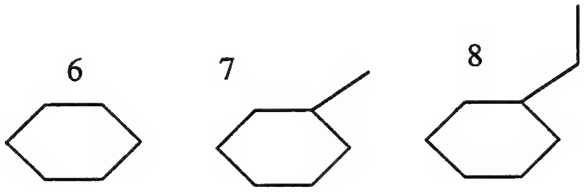
Paths



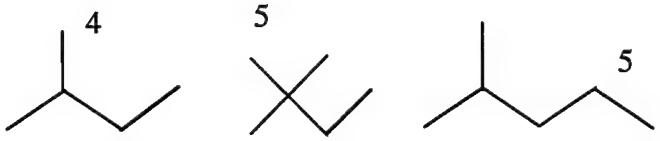
Clusters



Chains



Paths/Clusters



levels are represented by ${}^{n}X$ and valence levels ${}^{n}X^{v}$. Equation 13 is used to compute the zeroth order connectivity index level for n verticies.

$$ {}^{0}X = \sum c_i = \sum_{i=1}^{n} (\delta_i)^{-1/2} \tag{13} $$

The equation for the first order connectivity index, a single bond, is shown in Equation 14.

$$ {}^{1}X = \sum c_{ij} = \sum_{s} (\delta_i \delta_j)_s^{-1/2} \tag{14} $$

Subsequent orders for the subgraph "c" terms are represented by the subscripts k, l, m, n, and so on for subsequent connectivity levels. Paths, clusters, and chains are denoted by adding subscripts to the chi value (X_p, $X_{c,}$, and X_{ch}). The precise methodology of calculating the X indices is beyond the scope of this report and is fully reported in Reference 57. The resultant molecular connectivity chi terms are therefore structural descriptors that are unique for a given molecular structure and encode the number of atoms, number and degree of connections, unsaturation, heteroatom type and position(s), and cyclization. Computer programs have been developed to determine the X and X^v values for specific compounds (Graph III or TOPKAT [Reference 58] for example). Computer programs have also been developed to perform the subsequent regression analyses needed to establish relationships between molecular structure and various physiochemical properties.

2. Use of Molecular Connectivity Indices

Each of the ${}^nX$ and ${}^nX^v$ indices is uniquely calculated for an atom according to the description above. In general, a matrix of the biological activity values (which typically are in a logarithmic format) and the X values determined to be of the most usefulness is set up and solved by standard methods. Each of the X values corresponds to a certain feature of the molecule. Reference 57 describes how each of the X values may be used in this manner.

Equations may contain a mixture of X indices and be of the form

$$a = b + c\,{}^2X + d\,{}^6X_p + e\,{}^0X^v \qquad (15)$$

where a may be in logarithmic form, and b, c, d, and e may be positive or negative. While any appropriate X or X^v index may be used, many X indices contain the same information, and the choice of appropriate X indices may not always be independent. In some cases, the difference or sum of X indices, primarily between ${}^nX$ and ${}^nX^v$ may prove useful in determining the best equation.

The choice of X indices in the search for the best variables in a regression analysis is among the most critical in the QSAR process. Many indices have the same information embedded within them, and the use of only these indices would lead to a poor correlation.

Investigation of the structure of the molecules involved may also help in the choice of X indices; the higher X indices of many molecules are identical and their use would not lead to a higher quality correlation. Abbreviation of part of the structure in large molecules may be helpful. Often indices related to the terminal groups may prove to be the most useful, as they may be the only ones that differ from molecule to molecule. However, severe truncation to a model containing only the variable features of a part of the chain may result in the decline of the quality of the correlation. From Reference 57, a general rule might be to truncate a large structure to retain major structural features that may play a role in interacting with the variable part of the molecule.

Many QSAR analyses use both physical descriptors and X indices to provide the equation. Reference 57 discourages this, because all or most of the information conveyed by the physical descriptors is imbedded within the X indices.

Because of the different δ^v values for halogen heteroatoms, it possible to construct equations based on X^v that reflect the differences between chlorinated or fluorinated molecules, many of that have undergone toxicity testing, and brominated molecules, which are of interest as replacements to halons in firefighting applications and may not have undergone toxicity testing.

D. LINEAR SOLVATION ENERGY RELATIONSHIPS

Linear Solvation Energy Relationship (LSER) analysis is based on the correlation of diverse chemical properties, including toxicity, to solvent-solute interaction. The analysis is based on four molecular parameters: the intrinsic molecular volume, polarity, and two measures of the ability to participate in hydrogen bonding as an acceptor or donor. While this measure provides the most accurate QSARs covering the widest range of chemical classes, the parameters are available on only a finite amount of chemicals (Reference 47).

E. MIXTURES

Estimation methods for the biological activity of a mixture, when the activities of the separate components are known, are discussed in Reference 59. The estimation methods depend on the type of joint action. When two or more chemicals act independently with separate effects (independent, dissimilar), the results can be written as a probability of observing a given endpoint. Thus, if the probability of a particular endpoint (say death) is $P_1(d_1)$ for a dose d_1 of chemical $_1$ and $P_2(d_2)$ for a chemical 2, the probability of that endpoint for simultaneous administration those doses of both chemicals, $P(d_1,d_2)$, is given by Equation 13.

$$P(d_1,d_2) = P(d_1) + P(d_2) - P_1(d_1)P_2(d_2) \tag{16}$$

The more interesting case occurs when there is similar joint action by two or more chemicals operating independently (independent, similar). An appropriate example is when two or more halocarbons cause cardiac sensitization and one chemical does not affect another. Given a mixture of n chemical containing fractions f_i of chemicals i, where $\sum_{i=1}^{n} f_i = 1$, with endpoint values E_i (e.g., LD_{50}, NOAEL, etc.), the endpoint value E for the mixture is estimated from Equation 14.

$$\frac{1}{E} = \sum_{i=1}^{n} \frac{f_i}{E_i} \tag{17}$$

The above cases are for independent action by the components. When the action is dependent (e.g., synergism), one cannot statistically predict the effect of a mixture without more information about the mechanisms involved.

SECTION V
RECOMMENDATIONS

Two steps are needed to start screening chemicals:

1. A literature search for *in vitro* toxicity data for the chemicals of interest and members of similar families should be undertaken. This has been started in a companion document.*

2. Each chemical and chemical family of materials being considered as a potential replacements for Halon 1211 should be investigated using QSAR techniques to determine whether toxicity characteristics of the chemical or chemical family exist that would be unacceptable as a replacement. A list of chemicals identified as being potential halon replacements should be developed, and analogs for each chemical determined. A detailed search should be made for all toxicological data on the identified chemicals and their analogs. These data should be analyzed to determine whether sufficient information is available to proceed with QSAR toxicity analysis and to determine the format (property descriptors or molecular connectivity) for the analysis.

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APPENDIX A

GLOSSARY

Aliphatic—Related to straight-chain, branched-chain, or cyclic hydrocarbons or hydrocarbon derivatives containing carbon atoms linked by single sp^3-sp^3 bonds.

Aromatic—Cyclic molecules or fused cyclic molecules containing formally alternating single and double bonds with delocalized π electrons. The most common simple aromatic compound is benzene.

Arrhythmia—An irregular heartbeat.

Asphyxia—A local or systemic oxygen deficiency and an excess of carbon dioxide in a living organism as can be induced by an interruption of respiration.

Atrioventricular—Related to an atrium and a ventricle or between an atrium and a ventricle.

Atrium—A chamber of the heart that receives blood from the veins and passes it to a ventricle.

Bigeminy—Two quick heartbeats with a pause following each pair as in ventricular bigeminy.

Cardiac—Having to do with the heart.

Chlorofluorocarbon—An aliphatic compound containing only chlorine, fluorine, and carbon. An example is CFC-12, CCl_2F_2.

Contractility—The capability of contracting as in myocardial contractility (the contractility of the heart myocardium)

Cyclic—Arranged in a closed ring rather than an open chain.

Empirical Formula—A formula that gives the numbers of each type of atom without regard to structure. Isomers of the same compound will all have the same empirical formula.

Ester—A compound formed from an alcohol and an oxyacid by elimination of water.

Halocarbon—A compound of carbon and one or more halogen atoms with or without hydrogen.

Halogen—One of the elements or atoms fluorine (F), chlorine (Cl), bromine (Br), iodine (I), and astatine (At). Astatine, a radioactive element, is of no interest here.

Hammett Constant, σ_x—A constant that can be applied to a substituent group that determines its effect on a reaction equilibrium or a reaction rate at a site remote from the substituent. Hammett Constants are measures of electron withdrawl or electron induction. Hammett Constants are normally appropriate only for stereochemically rigid systems such as aromatic rings.

Hansch Equation—An equation relating biological activity to hydrophobic, electronic, and steric factors. It is commonly used for QSAR correlations.

Hydrocarbon—A binary compound of carbon and hydrogen. This term includes aliphatic compounds (paraffins), compounds containing double bonds (olefins), and aromatics.

Hydrochlorofluorocarbon—An aliphatic compound containing only hydrogen, chlorine, fluorine, and carbon. An example is HCFC-123, $CHCl_2CF_3$.

Hydrofluorocarbon—An aliphatic compound containing only hydrogen, fluorine, and carbon. An example is HFC-134a, CH_2FCF_3.

Hydrolysis—The reaction of a material with water. Examples are the reaction of a silicon halide with water, $SiR_3Cl + H_2O \rightarrow SiR_3OH + HCl$, and of an ester with water, $RCOOR' + H_2O \rightarrow RCOOH + R'OH$.

In vitro—Studies or methods that do not involve the use of live animals.

In vivo—Studies or methods which involve the use of live animals.

Isomer—A compound containing a specific arrangement of atoms (structure) making it differ from another compound with the same empirical formula, but with a different structure.

Molar Refractivity—$M_R = \frac{M(n_D^2 - 1)}{d(n_D^2 + 2)}$, where n is the refractive index, d is the density, and M is the molecular weight.

Molecular Connectivity Descriptors—Composite description of all structural features of a compound. They are most often presented as Molecular connectivity indices (MCI) which associate numerical values with the structure of the molecule.

Myocardium—The middle muscular layer of the heart wall.

Myocardial—Relating to the Myocardium.

Ouabain—The highly toxic glucoside g-strophanthin, $C_{29}H_{44}O_{12}\cdot 8H_2O$.

Partition Coefficient—The equilibrium ratio of the concentrations of a solute in two immiscible liquid phases. For example, the octanol/water partition coefficient is given by $K_{OW} = [C]_{octanol}/[C]_{water}$, where $[C]_s$ denotes the solute concentration in the solvent s.

Perfluorocarbon—An aliphatic compound containing only fluorine and carbon. An example is FC-218 (sometimes called PFC-218), $CF_3CF_2CF_3$.

Purine—One of a number of basic compounds having a structure similar to that of imidazo (4,5-d) pyrimidine, the bicyclic compound CHNCHNCCNHCHN. Examples are adenine, caffeine, guanine, hypoxanthine, theobromine, uric acid, and xanthine.

Pyrimidine—A group of basic compounds that can be isolated from nucleic acid hydrolysis. Examples are cytosine, methylcytosine, thymine, and uracil.

Quantitative Structure-Analysis Relationships (QSAR)—Correlations between a biological activity and physical, chemical, and structural composition of a compound. QSARs are used to determine the toxicity of an unknown substance based on the measured toxicity of similar chemicals.

Refractive Index—Ratio of velocity of light in a vacuum to that in a given material.

Tachycardia—Rapid heartbeat as in ventricular tachycardia.

Taft Polar Substituent Constant, σ^*—A constant closely related to the Hammett Constant but derived from acid- and base-catalyzed hydrolysis rates of substituted aliphatic esters.

Taft Steric Substituent Constant, E_s—A measure of the steric effect of a substituent in a molecule.

Ventricle—A chamber of the heart receiving blood from an atrium and pumping it into arteries.

Ventricular—Having to do with a heart ventricle.

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